A Novel Variation of *PLAGL1* in Chinese Patients with Isolated Ventricular Septal Defect

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Aims: Ventricular septal defect (VSD) is the most common congenital heart disease (CHD). A number of genetic studies have linked the gene of *PLAGL1* to the etiology of CHD. The present study aimed to identify potential pathogenic mutations for *PLAGL1* and to provide insights into the etiology of isolated VSD. **Methods:** Case-control mutational analysis was performed in 300 patients with isolated VSD and 300 healthy controls. Two protein-coding exons of *PLAGL1* and their partial flanking intron sequences were amplified by polymerase chain reaction and sequenced on an ABI3730 Automated Sequencer. CLC workbench software was used to compare the conservatism of *PLAGL1* protein with other multiple species. **Results:** Neither missense nor frame-shift mutations were detected in two protein-coding exons of *PLAGL1*. But a novel synonymous variation (c.486A>G, p. E162E) was detected in protein-coding exon-2. The glutamic that translated with the mutational codon is conservative when compared with other species. **Conclusions:** We detected a synonymous variation in the protein-coding exon-2 of *PLAGL1* in isolated VSD patients. It is possible that the etiology of isolated VSD might not be directly linked with this mutation, but might be associated with other patterns of gene expression regulation in *PLAGL1*, such as in the methylation-dependent manner.

Introduction

VENTRICULAR SEPTAL DEFECT (VSD) is the most common congenital heart disease (CHD). Studies have suggested that up to 4% of asymptomatic neonates, at birth, had VSD (Ferencz *et al.*, 1985; Hiraishi *et al.*, 1992). The causes of VSD are largely unknown. However, epidemiologic studies reveal a significant environmental contribution to the pathogenesis of VSD (Sands *et al.*, 1999; Burki and Babar, 2001; Burdan *et al.*, 2006). Familial aggregation and twin studies indicate the presence of genetic factors for susceptibility to this condition (Seides *et al.*, 1979; Maestri *et al.*, 1988; Oyen *et al.*, 2010).

Imprinting is a non-Mendelian form of inheritance in which only one of the two inherited alleles is expressed (Reik and Walter, 1998; Li and Sasaki, 2011). Approximately, 40 human genes regulated by imprinting have been reported, many of which, however, have not been fully characterized (Kamiya *et al.*, 2000). It has become clear that imprinting is involved in a number of human genetic disorders, such as Beckwith-Wiedemann syndrome (BWS), Prader-Willi syndrome, Angelman syndrome, and Wilm' tumor. *PLAGL1* (PLAG-like 1; also called *Lot1* or *Zac1*) is a maternally imprinted gene that belongs to an

imprinting cluster located on chromosome 6q24 (Arima *et al.*, 2006; Diplas *et al.*, 2009). It encodes a zinc finger protein that is thought to function as a transcription factor inducing apoptosis and cell cycle arrest at G1 phase (Bilanges *et al.*, 2001). In addition, there is also evidence that indicates its crucial regulation by epigenetic-factor-mediated chromatin rearrangements and alternative promoter usage (Valleley *et al.*, 2007).

The protein of *ZAC1* (*PLAGL1*) is an essential cardiac transcriptional factor, being highly expressed in mouse hearts from embryonic day (E) 8.5 to adulthood. *Nkx2.5* and *ZAC1* bind to adjacent sites within the atrial natriuretic peptide promoter, which physically interacts, and synergistically activates cardiac gene expression. To investigate the *in vivo* function of *ZAC1*, Yuasa *et al.* (2010) generated transgenic *Zac1*-null mice and induced defective embryonic heart development and reduced the expression of chamber and myofilament genes.

6q24-related transient neonatal diabetes mellitus (6q24-TNDM) is caused by the overexpression of paternally expressed imprinted genes at 6q24, in the position of which *PLAGL1* and *HYMAI* are identified. 6q24-TNDM caused by generalized hypomethylation at imprinted loci can be

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associated with marked CHD, deafness, epilepsy, and renal malformations (Temple and Mackay, 1993; Delaval *et al.*, 2006).

From the previous discussion, we predict that the gene of *PLAGL1* may play an important role in the development of VSD. The aims of our study are to identify potential pathogenic mutations for *PLAGL1* and to provide insights into the etiology of isolated VSD.

Patients and Methods

Patients

Blood samples were recruited from a total of 300 patients with isolated VSD and 300 controls with the same ethnic, gender, age, and no reported cardiac phenotype. Sample collection was approved by the ethics committee of TEDA International Cardiovascular Hospital, Tianjin, China. Informed consent form was obtained from their parents or guardians.

Clinical assessment of the patients was performed, which includes anthropometric measurement, physical examination for dysmorphism and malformation, and radiological evaluation. The patients also underwent chest X-ray examination, electrocardiogram, and ultrasonic echocardiogram. In this study we only enrolled those patients who were confirmed with isolated VSD without any other diseases possibly found by the examinations just outlined and during the heart surgery.

DNA extraction and mutation analysis

Genomic DNA was extracted from peripheral blood leukocytes by QIAamp blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Then, the genomic DNA was tested by 1% agarose gel and NanoDroop 2000 instrument. The DNA was stored at -20°C before use.

The two protein-coding exons of the *PLAGL1* gene and their partial flanking intron sequences were amplified by polymerase chain reaction (PCR) with two pairs of *PLAGL1* gene-specific primers (1F: 5'-GCTGAGAGGTCCATGTC TGG-3', 1R: 5'-AACCACAGGAATCACTGGATG-3', 1485 bp; 2F: 5'-TGGGCAAAAGAGTCAGATCC-3', 2R: 5'-GGCAC AATACATGCAGTTCG-3', 1655 bp). The PCR primers were designed by using GeneTool Software.

The PCR products were sequenced on an ABI3730 Automated Sequencer (PE Biosystems, Foster City, CA). The data were compared with sequences from the NCBI GenBank (*PLAGL1*: NM_001080951.1).

CLC workbench software was used to compare the conservatism of *PLAGL1* protein with other multiple species.

Results

A novel synonymous variation of *PLAGL1* in patients with isolated VSD

Neither missense mutation that causes substitutions of one amino acid to another amino acid, nor frame-shift mutations that are resulted from the shift of splice site were found in the study. But a novel synonymous variation (c.486A>G, p. E162E) was found in protein-coding exon-2 through the sequencing analysis of the *PLAGL1* gene in 300 patients with isolated VSD (Fig. 1) while none in healthy controls.

FIG. 1. A synonymous variation (p. E162E) in the protein isoform 2 of *PLAGL1* is identified in this study and is indicated by an arrow (upper). Electropherogram of the identified variation at the position 486 (A>G) of *PLAGL1* gene as an A/G double peak is shown by an arrow (lower).

The novel variation in this study resulted in a substitution of GAA to GAG in the codon of the glutamic (Glu) residue in the position 162 of *PLAGL1* protein isoform 2, but this change in DNA does not lead to an amino acid residue alteration.

The comparison of conservatism of *PLAGL1* protein among multiple species

The multiple alignment of amino acid sequence of the *PLAGL1* gene was taken from different species. The variation of DNA resulted in a synonymous variation. The position 162 in the *PLAGL1* protein isoform 2 was conservative among many species (Fig. 2) and suggested that it might play an important role in maintaining of the protein function. As shown in the Figure 2, the amino acid in position 162 in cattle and house mouse is Asp, while in human, pig, and Norway rat it is Glu. However, these two amino acids in this position are all acidic amino acid.

Discussion

CHD is the most frequently occurring congenital disorder in newborns as well as the most frequent cause of infant death

FIG. 2. Multiple-sequence alignment of partial amino acid sequence of *PLAGL1* protein in different species is shown. The alignment data indicate that glutamate at position 162 (indicated by an arrow) is conserved in different species in the *PLAGL1* protein.

from birth defects (Samanek *et al.*, 1999; Van der Bom *et al.*, 2011). A small proportion of CHD that occurs is associated with single-gene Mendelian disorders, chromosomal anomalies, and toxic or infective exposures during pregnancy (Lamy *et al.*, 1957). However, the etiology of many CHD, no matter as single disease or being associated with other malformations, is still unidentified and usually attributed to unspecified combinations of environmental and genetic causes (Reamon-Buettner and Borlak, 2005; Liu *et al.*, 2009).

PLAGL1 is a protooncogene and a target of chromosomal rearrangements that result in tumorigenesis. It contains eight exons (two protein-coding exons) and spans 125 kb (Valleley *et al.*, 2007). As a maternally imprinted gene, it is only expressed in the paternal allele in many tissues (Jager *et al.*, 2009). To investigate the *in vivo* function of *PLAGL1*, Yuasa *et al.* (2010) generated transgenic *PLAGL1*-null mice and found partial embryonic lethality and cardiac malformation in *PLAGL1*(*Zac1*)-null embryos. In addition, the *PLAGL1* (*Zac1*) mutant mouse shows abnormal cardiac gene expression and patterning as well as a significantly increased number of apoptotic cells in the heart (Yuasa *et al.*, 2010). Therefore, we believe that *PLAGL1* (*Zac1*) plays an essential role in the cardiac gene regulatory network and is an indispensable gene in the development of mice heart. Interestingly, in our study, we did not find any missense mutations and frame-shift mutations of *PLAGL1* in patients with isolated VSD. This might be due to the fact that our patient group was different from some other groups with VSD and associated diseases, such as obstruction of the right ventricular outflow tract, or complications, such as severe pulmonary hypertension. In fact, our study was designed to identify the genetic etiology of isolated VSD and therefore the pathology of our patients was relatively mild compared with other groups of VSD patients mentioned earlier.

It has been reported that the methylation of the gene *PLAGL1* is linked with some congenital diseases (Arima *et al.*, 2005; Blik *et al.*, 2009). For example, BWS is characterized by omphalocele, macroglossia, adrenal cortical cytomegaly, and hypoglycemia in the neonatal period. Cardiac malformations of this syndrome contain cardiomegaly or congenital anomalies, such as VSD, atrial septal defect, and anomalous retroesophageal right subclavian artery (Kapur *et al.*, 1985). Studies have demonstrated that the gene *PLAGL1* is involved in the etiology of BWS. The protein of *PLAGL1* may regulate p57^{KIP2} through LIT1 (KCNQ1OT1) (methylation-dependent manner), which forms part of a novel signaling pathway to regulate cell growth. Another example is 6q24-TNDM in which generalized hypomethylation at imprinted loci (*PLAGL1* and *HYMAI*) might be associated with marked CHD, deafness, epilepsy, and renal malformations (Temple and Mackay, 1993). Therefore, there is a possibility that methylation of *PLAGL1* might be linked with development of VSD.

Two limitations of this study should be discussed. First, modern genetics demonstrated that the introns and noncoding exons might play an important role in the gene expression regulation (Ren, 2010). In our study, only two protein-coding exons and their partial flanking sequences of *PLAGL1* were amplified and sequenced. Therefore, our study does not rule out the role of introns and noncoding exons of *PLAGL1* in the development of isolated VSD. Second, this study only investigated the Chinese population. The relationship between

PLAGL1 and isolated VSD in other populations should also be revealed in the future.

In summary, in the present study the mutational analysis of *PLAGL1* in 300 Chinese patients with isolated VSD revealed a novel synonymous variation (c.486A>G, p. E162E). It is possible that the etiology of isolated VSD might not be directly linked with this variation, but might be associated with other patterns of gene expression regulation in *PLAGL1*, such as in the methylation-dependent manner.

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Author Disclosure Statement

No competing financial interests exist.

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